REMARKS

In the Office Action dated January 25, 2007, claims 1, 3, 4, 6, 7, 9-12 and 14, in the above-identified U.S. patent application were rejected. Reconsideration of the rejections is respectfully requested in view of the above amendments and the following remarks. Claims 1, 3-4, 6, 7, 9-12 and 14 remain in this application, claims 2, 23 and 24 have been canceled and claims 5, 8, 13, and 15-22 have been withdrawn.

The office action indicates that the non-elected claims should be canceled. Claims 23 and 24 directed to an antigen have been canceled. Claims 5, 8, 13, and 15-22 have not been canceled as applicant's understanding is that these claims could be rejoined if the elected claims are found allowable.

Claims 1, 3-4, 9-12 and 14 were rejected under 35 USC §103(a) as unpatentable over Hashida and Formoso. Applicants respectfully contend that neither Hashida nor Formoso suggests or discloses a first antigen for an antibody, which has at least one marker group, and comprises multiple epitope regions, said epitope regions being identical in amino acid sequence. In addition, neither of these references suggest or discloses a method where the first and second antigens are identical as in claim 4. Hashida uses a mixture of different antigens or their conjugates (both p17 and p24 are used) in his method (see table 2, right hand column). Included with the present response is a paper by Hashida, Journal of Clinical Laboratory Analysis, 8:86-95 (1994). As can be gathered from page 88, first column, under the headline "Recombinant p24-p-galactosidase conjugate", a conjugate which was already known in the art is applied by Hashida. The conjugate mentioned in Hashida contained an average of 2.1 p24 molecules per molecule R-D-galactosidase. Therefore, the detection

reagent does not contain a carrier molecule. The claims have been amended to recite that at least the first antigen has the recited formula. Applicants point out that the first antigen is the detection antigen and the second antigen is the capturing antigen. In contrast to this, the office action does not differentiate between the capturing and detection antigens applied by Hashida. The office action states that "the antigen being conjugated (e.g. covalently bound) to both BSA (e.g. carrier) and enzyme labels (e.g. marker group)..." but does not acknowledge that the conjugates of DNP, BSA and recombinant HIV antigen are exclusively applied as capturing antigens (i.e. they correspond to the "second antigen" of the present application).

Applicants also point out that Hashida is an ultra sensitive immune complex transfer enzyme immunoassay wherein an immune complex is immobilized on a first solid phase and subsequently transferred to a second solid phase for detection. In contrast to this, in the present invention, detection and immobilization take place on the same solid phase. Though other methods were known in the art, one would not carry out Hashida's method without transfer to a second solid phase as this would destroy the point of Hashida's method. Hashida shows that the advantage of the method described therein is the transfer of immune complexes from the anti-DNP solid phase to an anti-IgG solid phase. In contrast to Hashida, the present invention uses an antigen with several identical epitope regions to improve the sensitivity of the test.

Formoso does not cure the deficiencies in Hashida as Formoso does not suggest or disclose a detection reagent (i.e. first antigen) with the formula recited in claim 1. Applicants contend that even if one were to combine Hashida and Formoso, the combination would not suggest a method where the detection antigen has the formula recited in claim 1 and where the detection and immobilization take place on the same solid phase. In addition, Formoso

does not suggest a method where the first and second antigens are identical as in claim 4. Since neither Hashida nor Formoso suggest or disclose the use of multimeric detection antigens with multiple, identical epitope regions, applicants request that this rejection be withdrawn.

Claims 6 and 7 were rejected under 35 USC §103(a) as unpatentable over Hashida and Formoso in view of Watts. Watts was cited for the disclosure of antidigoxigenin and antidigoxigenin antibody in binding assays. Watts does not disclose a detection antigen with multiple identical epitope regions and thus does not cure the above discussed deficiencies in Hashida and Formoso. The presently claimed invention uses a first (detection) antigen with several identical epitope regions which improves the sensitivity of the test. Since neither Hashida, Formoso, nor Watts disclose the use of multiple identical epitope regions in a detection antigen, applicants contend that the presently claimed invention is patentable over the combination of Hashida, Formoso and Watts and request that this rejection be withdrawn.

Applicants respectfully submit that all of claims 1, 3-4, 6, 7, 9-12 and 14 are now in condition for allowance. If it is believed that the application is not in condition for allowance, it is respectfully requested that the undersigned attorney be contacted at the telephone number below.

In the event that this paper is not considered to be timely filed, the Applicant respectfully petitions for an appropriate extension of time. Any fee for such an extension together with additional fees that may be due with respect to this paper may be charged to Counsel's Deposit Account No. 02-2135.

Respectfully submitted,

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Detection of Antibody IgG to HIV-1 in Urine by Ultrasensitive Enzyme Immunoassay (Immune Complex Transfer Enzyme Immunoassay) Using Recombinant p24 as Antigen for Diagnosis of HIV-1 Infection

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Anti-HIV-1 IgG in urine was detected by an ultrasensitive enzyme immunoassay (immune complex transfer enzyme immunoassay) using recombinant p24 *gag* protein (p24) of HIV-1 as antigen and β-D-galactosidase from *Escherichia coli* as label. Anti-HIV-1 IgG in urine was reacted simultaneously with 2,4-dinitrophenyl-bovine serum albumin-recombinant p24 conjugate and recombinant p24-β-D-galactosidase conjugate. The complex formed, consisting of the three components, was trapped onto polystyrene balls coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG, eluted with εN-2,4-dinitrophenyl-L-lysine, and transferred to polystyrene balls coated with affinity-purified (anti-human IgG γ-chain) IgG. Bound β-D-galactosidase activity was assayed by fluorometry. This assay was at least 3,000-fold more sensitive than conventional methods. The lowest signal among 49 asymptomatic carriers was 3.1-fold higher than the highest nonspecific signal among 100 seronegative subjects. The sensitivity and specificity were both 100%. The positivity could be confirmed by preincubation of urine samples with excess of the antigen. Thus, this assay would be a powerful tool for detecting IgG antibody to HIV-1 in urine.

Key words: antibody, human immunodeficiency virus type 1, p24, β-D-galactosidase, ELISA, gelatin particle agglutination

INTRODUCTION

Antibodies to human immunodeficiency virus type 1 (HIV-1) in serum, plasma and whole blood have been detected by various methods such as enzyme-linked immunosorbent assay (ELISA), agglutination of latex, red-cells and gelatin particles, dot blotting, and Western blotting (1). Antigens used are the whole virus, recombinant proteins, and synthetic peptides (1). These tests are sufficiently sensitive for the diagnosis of HIV-1 infection (1). However, blood should be collected with due cautions to avoid infections not only with HIV but also with other pathogens. By contrast, urine can be collected more easily with no invasive procedures, less expenses, and less possibility of various infections than blood (2). Recently, a sensitive enzyme immunoassay (immune complex transfer enzyme immunoassay) using recombinant reverse transcriptase (RT) and p17 of HIV-1 (NL4-3) (3) as antigens and horseradish peroxidase and *Escherichia coli* β-D-galactosidase, respectively, as labels has been described for the detection of antibody IgG to HIV-1 in urine (4). This assay was at least 30-fold more sensitive than conventional methods. The sensitivity and specificity were both 100%. The positivity with one of the two antigens could be confirmed by using the other antigen and/or approximately 10-fold concentrated urine samples. Test results were considered to become more reliable by increasing the number of antigens such as RT and p17, with which the sensitivity and specificity are both 100%.

This paper describes the detection of anti-HIV-1 IgG in urine by the immune complex transfer enzyme immunoassay

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using recombinant p24 of HIV-1 (NL4-3) (3) as antigen and β-D-galactosidase from *Escherichia coli* as label.

MATERIALS AND METHODS

Buffers

The regularly used buffers were 0.1 mol/L sodium phosphate buffer, pH 6.0, containing 5 mmol/L EDTA (buffer A); and 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 g/L bovine serum albumin (fraction V, Intergen Company, Purchase, NY) and 1.0 mmol/L $MgCl_2$ and 1.0 g/L NaN_3 (buffer B).

Antibodies

Rabbit (anti-2,4-dinitrophenyl-bovine serum albumin) serum was obtained from Shibayagi Co., Ltd., Gumma, Japan. Rabbit (anti-human IgG γ-chain) IgG was obtained from Medical and Biological Laboratories Co., Ltd., Nagoya, Japan. IgG was prepared from serum by fractionation with Na_2SO_4 followed by passage through a column of DEAE-cellulose (5). The amount of IgG was calculated from the absorbance at 280 nm (5).

2,4-Dinitrophenyl-Bovine Serum Albumin

Thiol groups were introduced into bovine serum albumin molecules using *N*-succinimidyl-*S*-acetylmercaptoacetate and were reacted with maleimide groups introduced into ε*N*-2,4-dinitrophenyl-L-lysine molecules using *N*-succinimidyl-6-maleimidohexanoate (6). The amount of bovine serum albumin, 2,4-dinitrophenyl groups and 2,4-dinitrophenyl-bovine serum albumin was calculated from the absorbance at 280 and 360 nm (6). The average number of 2,4-dinitrophenyl groups introduced per albumin molecule was 6.0.

Protein-Sepharose 4B

2,4-Dinitrophenyl-bovine serum albumin (10 mg) and human IgG (10 mg) were coupled to CNBr-activated Sepharose 4B (1.0 g, Pharmacia LKB Biotechnology AB, Uppsala, Sweden) according to the instructions of Pharmacia LKB Biotechnology.

Affinity-Purification of Antibodies

(Anti-2,4-dinitrophenyl-bovine serum albumin) IgG and (anti-human IgG γ-chain) IgG were affinity-purified by elution at pH 2.5 from columns of 2,4-dinitrophenyl-bovine serum albumin-Sepharose 4B and human IgG-Sepharose 4B, respectively (7).

Protein-Coated Polystyrene Balls

Polystyrene balls (3.2 mm in diameter, Immuno Chemical, Inc., Okayama, Japan) were coated by physical adsorption with affinity-purified (anti-2,4-dinitrophenyl-bovine serum albumin) IgG (0.1 g/L) and affinity-purified (anti-human IgG γ-chain) IgG (0.1 g/L) (8). Affinity-purified (anti-2,4-dinitrophenyl-bovine serum albumin) IgG-coated polystyrene balls had been colored pink for discrimination from affinity-purified (anti-human IgG γ-chain) IgG-coated polystyrene balls.

Recombinant p24 of HIV-1

Recombinant p24 of HIV-1 (NL4-3) (3) was produced in *Escherichia coli* transformed with expression plasmid carrying the corresponding cDNA and purified as described previously (9). The purified recombinant p24 was found to be homogeneous by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

2,4-Dinitrophenyl-Bovine Serum Albumin-Recombinant p24 Conjugate

6-Maleimidohexanoyl-2,4-dinitrophenyl-bovine serum albumin

2,4-Dinitrophenyl-bovine serum albumin prepared as described above (3.0 mg, 45 nmol) in 0.49 ml of 0.1 mol/L sodium phosphate buffer, pH 7.0, was incubated with 10 µl of 20 mmol/L *N*-succinimidyl-6-maleimidohexanoate (DOJINDO Laboratories, Kumamoto, Japan) in *N,N*-dimethylformamide at 30°C for 30 min. After incubation, the reaction mixture was subjected to gel filtration by the centrifuged column procedure (10) using a column (1.1× 5.3 cm) of Sephadex G-50 fine (Pharmacia LKB Biotechnology AB), equilibrated with buffer A. The average number of maleimide groups introduced per albumin molecule was 3.6.

Mercaptoacetyl-recombinant p24

Recombinant p24 (1.6 mg, 67 nmol) in 0.48 ml of 0.1 mol/L sodium phosphate buffer, pH 7.0, was incubated with 20 µl of 5 mmol/L *N*-succinimidyl-*S*-acetylmercaptoacetate (Boehringer Mannheim GmbH, Mannheim, Germany) in *N,N*-dimethylformamide at 30°C for 30 min. After incubation, the reaction mixture was incubated with 30 µl of 0.1 mol/L EDTA, pH 7.0, 60 µl of 1 mol/L Tris-HCl buffer, pH 7.0, and 70 µl of 1 mol/L hydroxylamine, pH 7.0, at 30°C for 15 min and subjected to gel filtration on a column (1.0 × 45 cm) of Sephadex G-25 using buffer A. The concentration of recombinant p24 was determined by a commercial protein assay kit (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as standard, and its molecular weight was taken to be 24,000 (9).

The average number of thiol groups introduced per recombinant p24 molecule was 1.1.

2,4-Dinitrophenyl-bovine serum albumin-recombinant p24 conjugate

Mercaptoacetyl-recombinant p24 (0.46 mg, 19 nmol) in 0.25 ml of buffer A was incubated with 6-maleimidohexanoyl-2,4-dinitrophenyl-bovine serum albumin (0.32 mg, 4.8 nmol) in 70 μl of buffer A at 4°C for 20 h. After incubation, the reaction mixture was incubated with 10 μl of 0.1 mol/L 2-mercaptoethylamine in buffer A at 30°C for 15 min and subsequently with 20 μl of 0.1 mol/L N-ethylmaleimide in buffer A at 30°C for 15 min. The reaction mixture was subjected to gel filtration on a column (1.5 × 45 cm) of Ultrogel AcA 44 (IBF Biotechnics, Villeneuve-la-Garenne, France) using 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 mol/L NaCl. The average number of recombinant p24 molecules conjugated per albumin molecule was 2.5, which was calculated from the concentration of 2,4-dinitrophenyl-bovine serum albumin and the total protein concentration determined by the commercial protein assay kit as described above. The amount of the conjugate was calculated in the same way as described for 2,4-dinitrophenyl-bovine serum albumin.

Recombinant p24-β-D-Galactosidase Conjugate

Maleimide-β-D-galactosidase

Maleimide groups were introduced into β-D-galactosidase molecules from *Escherichia coli* by *N,N'-o*-phenylenedimaleimide (5). The average number of maleimide groups introduced per β-D-galactosidase molecules was 14 (5).

Mercaptoacetyl-recombinant p24

Mercaptoacetyl-recombinant p24 was prepared in the same way as described for the preparation of 2,4-dinitrophenyl-bovine serum albumin-recombinant p24 conjugate.

Recombinant p24-β-D-galactosidase conjugate

Mercaptoacetyl-recombinant p24 (0.19 mg, 8.0 nmol) in 0.5 ml of buffer A was incubated with maleimide-β-D-galactosidase (1.1 mg, 2.0 nmol) in 0.5 ml of buffer A at 4°C for 20 h. After incubation, the reaction mixture was incubated with 10 μl of 0.1 mol/L 2-mercaptoethylamine in buffer A at 30°C for 15 min and subsequently with 20 μl of 0.1 mol/L *N*-ethylmaleimide in buffer A at 30°C for 15 min. The reaction mixture was subjected to gel filtration on a column (1.5 × 45 cm) of Ultrogel AcA 22 (IBF Biotechnics) using buffer B containing 0.1 mol/L NaCl. Fractions containing the conjugate were stored at 4°C. The average number of recombinant p24 molecules conjugated per β-D-galactosidase molecule was 2.1, which was calculated from the decrease in the number of maleimide groups (5). The amount of the conjugate was calculated from β-D-galactosidase activity.

Immune Complex Transfer Enzyme Immunoassay for Anti-HIV-1 IgG

An aliquot (100 μl) of urine samples was incubated with 20 μl of nonspecific rabbit serum and 10 μl of buffer B containing 0.4 mol/L NaCl and 50 μg of inactive β-D-galactosidase (β-galactosidase-mutein, Boehringer Mannheim GmbH) at room temperature for 3 h. Inactive β-D-galactosidase was used to eliminate interference by anti-β-D-galactosidase antibodies (11). The amount of inactive β-D-galactosidase was calculated from the absorbance at 280 nm using the extinction coefficient for β-D-galactosidase (5). In experiments to confirm the presence of anti-HIV-1 p24 IgG, 15 pmol of recombinant p24 was added. In experiments to examine the effect of urine volumes, the volume of buffer B containing 0.4 mol/L NaCl and 50 μg of inactive β-D-galactosidase was increased to a total volume of 110 μl. In experiment to examine the effect of pH, 1 μl of urine and 109 μl of 10 mmol/L sodium phosphate buffer, pH 5.0–8.0, containing 0.4 mol/L NaCl, 0.1 g/L bovine serum albumin, 1.0 mmol/L $MgCl_2$, 1.0 g/L NaN_3, and 50 μg of inactive β-D-galactosidase were used. The reaction mixture (130 μl) was incubated with 20 μl of buffer B containing 0.4 mol/L NaCl, 100 fmol of 2,4-dinitrophenyl-bovine serum albumin-recombinant p24 conjugate, and 100 fmol of recombinant p24-β-D-galactosidase conjugate at room temperature for 3 h. To the reaction mixture, two colored polystyrene balls coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG were added, and the incubation was continued at room temperature overnight. After removing the reaction mixture, the colored polystyrene balls were washed twice by addition and aspiration of 2 ml of buffer B containing 0.1 mol/L NaCl and incubated with 150 μl of buffer B containing 0.1 mol/L NaCl and 1.0 mmol/L *εN*-2,4-dinitrophenyl-L-lysine and two white polystyrene balls coated with affinity-purified (anti-human IgG γ-chain) IgG at room temperature for 1 h. The colored polystyrene balls were removed, and the incubation was continued at room temperature for 2 h. The white polystyrene balls were washed as described above. β-D-Galactosidase activity bound to the white polystyrene balls was assayed at 30°C for 25 h by fluorometry using 4-methylumbelliferyl-β-D-galactoside as substrate (12). The fluorescence intensity was measured relative to 1×10^{-8} mol/L 4-methylumbelliferone in 0.1 mol/L glycine-NaOH buffer, pH 10.3, using 360 nm for excitation and 450 nm for emission analysis with a spectrofluorophotometer (F-3010, Hitachi, Ltd., Tokyo, Japan).

For testing serum, an aliquot (20 μl) of samples was incubated with 20 μl of nonspecific rabbit serum and 90 μl of buffer B containing 0.4 mol/L NaCl and 50 μg of inactive β-D-galactosidase at room temperature for 3 h and processed as described above.

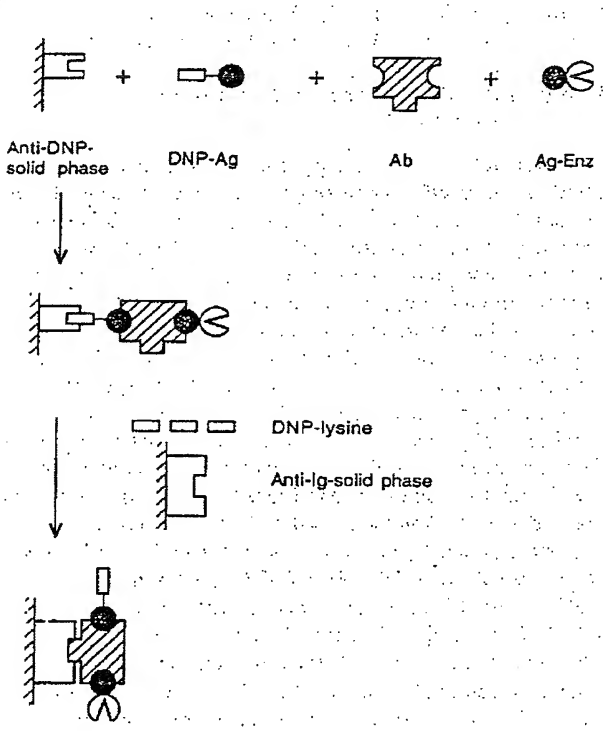

Fig. 1. Immune complex transfer enzyme immunoassay for antibody IgG. DNP: 2,4-dinitrophenyl group. Ag, antigen. Ab, antibody. Enz, enzyme. Ig, immunoglobulin.

Conventional Enzyme-Linked Immunosorbent Assay (ELISA) for Anti-HIV-1 IgG

The conventional ELISA was performed by using a commercial kit (ABBOTT HTLV-III EIA, ABBOTT Laboratories, North Chicago, IL). A polystyrene ball coated with five recombinant proteins of HIV-1 (gp120, gp41, p24, p17, and p15) was incubated with 100 µl of urine samples and 310 µl of the diluent included in the kit at 40°C for 30 min and, after washing, with (anti-human IgG) antibody-peroxidase conjugate at 40°C for 30 min. Bound peroxidase activity was assayed by colorimetry using 2-phenylenediamine as hydrogen donor.

Gelatin Particle Agglutination for Anti-HIV-1 Antibodies

Gelatin particle agglutination was performed using a commercial kit with a lysate of HIV-1 produced by MOLT #4/HTLV-III cell line as antigen (SERODIA-HIV, Fujirebio Inc., Tokyo, Japan) (13). Serum and urine samples were diluted at least 16-fold and 2-fold, respectively, with the diluent included in the kit. An aliquot (25 µl) of diluted samples was mixed with the particle solution (25 µl) in U-shaped wells of microplates and allowed to stand at room temperature for 2 h.

Western Blotting for Anti-HIV-1 IgG

Western blotting for anti-HIV-1 IgG was performed using a commercial kit (Ortho HIV Western Blot Kit, Ortho Diag-

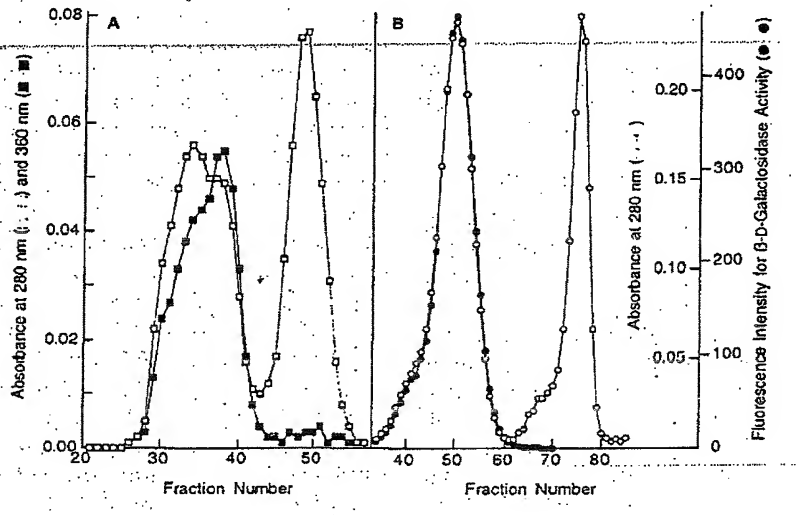

Fig. 2. Elution profiles of 2,4-dinitrophenyl-bovine serum albumin-recombinant p24 conjugate (A) and recombinant p24-β-D-galactosidase conjugate (B) from columns of Ultrogel AcA 44 and AcA 22, respectively.

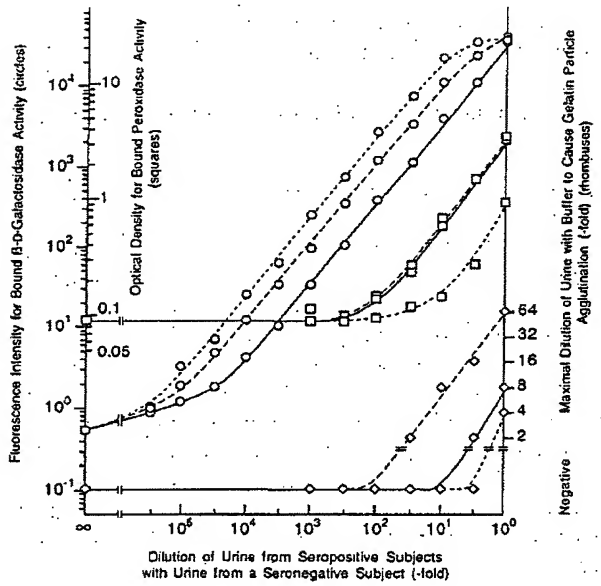

Fig. 3. Dilution curves of urine samples from seropositive subjects by different methods. Urine samples from three seropositive subjects were serially diluted with urine from a seronegative subject and were tested by the immune complex transfer enzyme immunoassay using recombinant p24 of HIV-1 as antigen and *Escherichia coli* β-D-galactosidase as label (open circles), the conventional ELISA using five recombinant proteins of HIV-1 as antigens (open squares) and gelatin particle agglutination using a lysate of HIV-1 as antigen (open rhombuses).

nostic Systems Inc., Raritan, NJ). Serum samples were diluted 101-fold with the diluent included in the kit. A nitrocellulose membrane preblotted with nine HIV-1 proteins (gp160, gp120, p66, p55, p51, gp41, p31, p24, and p17) was incubated with 2.02 ml of the diluted samples at room temperature overnight and, after washing, with biotinyl-(anti-human IgG) antibody at room temperature for 1 h. After washing, the nitrocellulose membrane was incubated with avidin-peroxidase conjugate at room temperature for 1 h. Bound peroxidase activity was detected using 4-chloro-1-naphthol as hydrogen donor.

Urine Samples From HIV-1 Seropositive and Seronegative Subjects

Urine samples were collected from 100 seronegative subjects aged 24–68 years and 70 seropositive subjects aged 10–60 years. Negative and positive sera were discriminated by gelatin particle agglutination. The seropositivity was confirmed by Western blotting. Urine samples collected were mixed with 1/100 volume of 10 g/L bovine serum albumin (fraction V, Intergen Company) and 1/100 volume of 100 g/L NaN_3 and stored at –20°C. Before use, urine samples were thawed and centrifuged at 1,500 × g at 4°C for 10 min to remove precipitates.

Immune Complex Transfer Enzyme Immunoassay for Anti-HTLV-I IgG

The immune complex transfer enzyme immunoassay for antibody IgG to human T-cell leukemia virus type (HTLV-I) was performed using 2,4-dinitrophenyl-bovine serum albumin-Cys-*env* gp46(188-224) conjugate and Cys-*env* gp46(188–224)-β-D-galactosidase conjugate essentially in the same way as the present enzyme immunoassay for anti-HIV-1 IgG (14). The volumes of serum and urine samples used were 20 μl and 100 μl, respectively. Rabbit serum (20 μl) was added for urine samples. Bound β-D-galactosidase activity was assayed for 2.5 h.

Gelatin Particle Agglutination for Anti-HTLV-I Antibodies

Detection of anti-HTLV-I antibodies by gelatin particle agglutination was performed using a commercial kit with HTLV-I produced by TCL-Kan cell line (15) as antigen (SERODIA-HTLV-I, Fujirebio, Inc.). Serum samples were diluted 8-fold or more with the diluent included in the kit. An aliquot (25 μl) of diluted serum was mixed with the particle

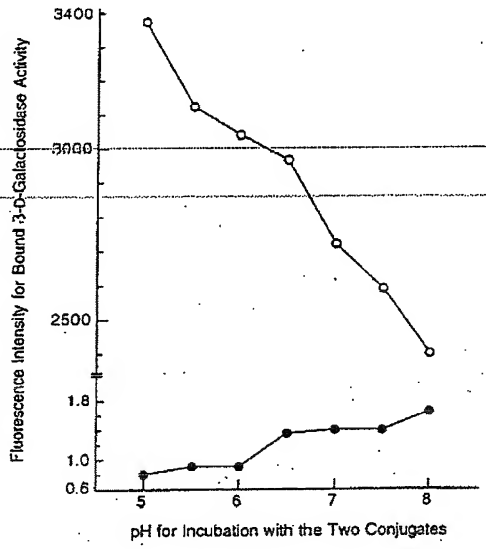

Fig. 4. Effect of pH in the immune complex transfer enzyme immunoassay for anti-HIV-1 IgG. Urine samples were incubated with 2,4-dinitrophenyl-bovine serum albumin-recombinant p24 conjugate and recombinant p24-β-D-galactosidase conjugate at pH 5–8. Open and closed circles indicate experiments with urine from a seropositive subject and a seronegative subject, respectively.

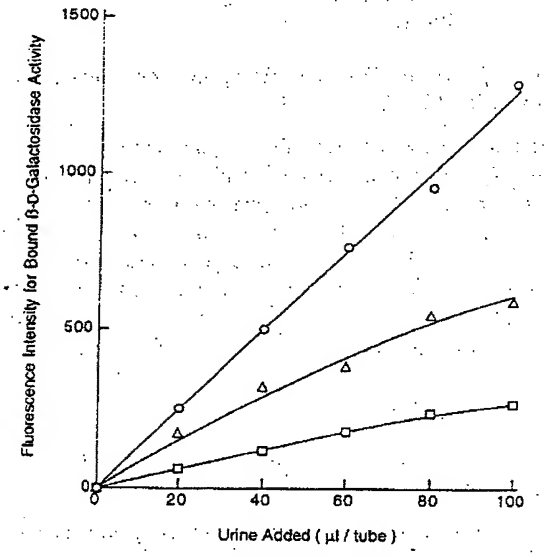

Fig. 5. Effect of urine volumes in the immune complex transfer enzyme immunoassay for anti-HIV-1 IgG. Increasing volumes of urine samples from three seropositive subjects were tested by the immune complex transfer enzyme immunoassay.

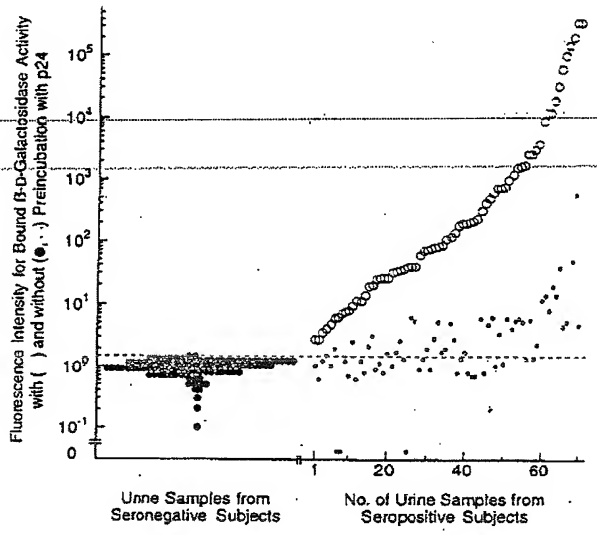

Fig. 6. Detection of anti-HIV-1 IgG in urine by the immune complex transfer enzyme immunoassay. Seventy urine samples from seropositive subjects and 100 urine samples from seronegative subjects were tested. Open and closed circles indicate signals for urine samples from seropositive and seronegative subjects, respectively. Open smaller circles indicate signals obtained by preincubation of urine samples with excess of recombinant p24. The broken line indicates a tentative cut-off value.

solution (25 μl) in U-shaped wells of microplates and allowed to stand at room temperature for 3 h.

Serum and Urine Samples From Subjects Infected With HTLV-I

Paired serum and urine samples were collected from 600 healthy subjects (382 males aged 19–77 years and 218 females aged 20–69 years). Serum samples were stored at –20°C. Urine samples were stored and used as described above. The serum samples were tested by the immune complex transfer enzyme immunoassay for anti-HTLV-I IgG and gelatin particle agglutination for anti-HTLV-I antibodies, and 31 serum samples (5.2%) out of the 600 were positive by both tests. The corresponding 31 urine samples were tested by the immune complex transfer enzyme immunoassay for anti-HTLV-I IgG. Ten urine samples out of the thirty-one, which showed higher signals than the other samples, and the corresponding ten serum samples were used for test by the immune complex transfer enzyme immunoassay for anti-HIV-1 IgG.

Measurement of Human IgG in Urine

Human IgG in urine was measured by two site enzyme immunoassay as described previously (16).

RESULTS AND DISCUSSION

Anti-HIV-1 IgG in urine was detected by an ultrasensitive enzyme immunoassay (immune complex transfer enzyme immunoassay) using recombinant p24 of HIV-1 (NL4-3) (3) as antigen and β-D-galactosidase from *Escherichia coli* as label (the immune complex transfer enzyme immunoassay) as shown schematically in Figure 1. 2,4-Dinitrophenyl-bovine serum albumin-recombinant p24 conjugate and recombinant p24-β-D-galactosidase conjugate were prepared by reacting thiol groups introduced into recombinant p24 molecules with maleimide groups introduced into 2,4-dinitrophenyl-bovine serum albumin and β-D-galactosidase molecules, and no significant polymerization was observed (Fig. 2). The results were compared with those by the conventional ELISA using five recombinant proteins of HIV-1 as antigens and gelatin particle agglutination test using a lysate of HIV-1 as antigen.

Sensitivity of the Methods Used

Three urine samples from seropositive subjects were serially diluted with urine from a seronegative subject and were tested by the above methods. The immune complex transfer enzyme immunoassay was approximately 3,000- to 300,000-fold more sensitive than the other methods (Fig. 3).

TABLE 1. Test Results of Urine Samples From 70 Seropositive and 100 Seronegative Subjects by the Immune Complex Transfer Enzyme Immunoassays, the Conventional ELISA, and Gelatin Particle Agglutination Test[a]

Method	Antigen	Sensitivity (%)	Specificity (%)	Lowest cut-off index		
				AC	ARC	AIDS
Immune complex transfer enzyme immunoassay	p24	100	100	3.1	2.1	2.1
	RT	100	100	1.5	1.4	1.1
	p17	100	100	3.1	2.6	1.9
Conventional ELISA	p15, p17, p24 gp41, gp120	91	99	0.3	0.7	1.0
Gelatin particle agglutination	HIV-1 lysate	83	97	—	—	—

[a]The lowest cut-off index is the ratio of the lowest signal for seropositive subjects to the highest nonspecific one for seronegative subjects. AC, 49 asymptomatic carriers. ARC, 13 patients with AIDS-related complex. AIDS, 8 patients with AIDS.

Interference by Urine

Effect of pH in the immune complex transfer enzyme immunoassay was examined by incubation of urine samples with 2,4-dinitrophenyl-bovine serum albumin-recombinant p24 conjugate and recombinant p24-β-D-galactosidase conjugate at pH 5.0–8.0, since pH of urine samples ranged from 5.3 through 8.0. The specific signal was higher at lower pH, and the specific signal at pH 5.0 was 1.4-fold higher than that at pH 8.0. The nonspecific signal was slightly higher at higher pH (Fig. 4). For effective detection of anti-HIV-1 IgG, adjustment of pH of urine samples to lower pH before use was recommended.

Increasing volumes of urine samples from seropositive subjects were tested by the immune complex transfer enzyme immunoassay. Up to 100 μl of urine samples could be used with only slight interference (Fig. 5).

Assay Variation

The assay variation in the immune complex transfer enzyme immunoassay was examined by using urine samples, which showed three different levels of the fluorescence intensity for bound β-D-galactosidase activity (14, 174, and 1,650 for within-assay and 16, 188, and 2,050 for between-assay). The number of determinations at each level was 10 for both within-assay and between-assay. The variation coefficients for within-assay and between-assay were 4.0–7.0% and 4.7–8.0%, respectively.

Detection of Anti-HIV-1 IgG in Urine

Seventy urine samples were collected from seropositive subjects aged 10–60 years (49 asymptomatic carriers, 13 patients with AIDS-related complex (ARC) and eight patients with AIDS), and 100 urine samples were collected from seronegative subjects aged 24–68 years. The seropositivity was confirmed by Western blotting. The concentration of urinary IgG tended to be higher in patients with AIDS than in the other subjects (4). These samples were tested by the immune complex transfer enzyme immunoassay (Fig. 6 and Table 1). All signals for the seropositive subjects were higher than those for the seronegative subjects. The lowest cut-off indices for the asymptomatic seropositive subjects and the patients with ARC and AIDS (the ratios of the lowest signals for the asymptomatic seropositive subjects and the patients with ARC and AIDS to the highest nonspecific signal for the seronegative subjects) were 3.1, 2.1 and 2.1, respectively.

Specificity

All signals for the seropositive subjects observed by the immune complex transfer enzyme immunoassay were significantly lowered by preincubation of urine samples with excess of recombinant p24 of HIV-1 (Fig. 6).

Ten paired serum and urine samples, which were strongly positive by the immune complex transfer enzyme immunoassay for anti-HTLV-I IgG, were tested by the immune complex transfer enzyme immunoassay for anti-HIV-1 IgG. A serum sample reacted slightly, but none of the other samples reacted (Fig. 7).

Level of Anti-HIV-1 IgG in Different Stages of HIV-1 Infection

Levels of anti-HIV-1 p24 IgG were significantly ($P < 0.001$) lower in the patients with ARC and AIDS than in the asymptomatic carriers (Fig. 8), which was consistent with previous reports (17–19).

Comparison With Previous Reports

Anti-HIV-1 antibodies in urine were detected previously by the conventional ELISA (20–22), IgG antibody-capture enzyme-linked immunosorbent assay (GACELISA) (23–25) and IgG antibody-capture particle adherence test (GACPAT) (23, 24). The sensitivity and specificity of the conventional

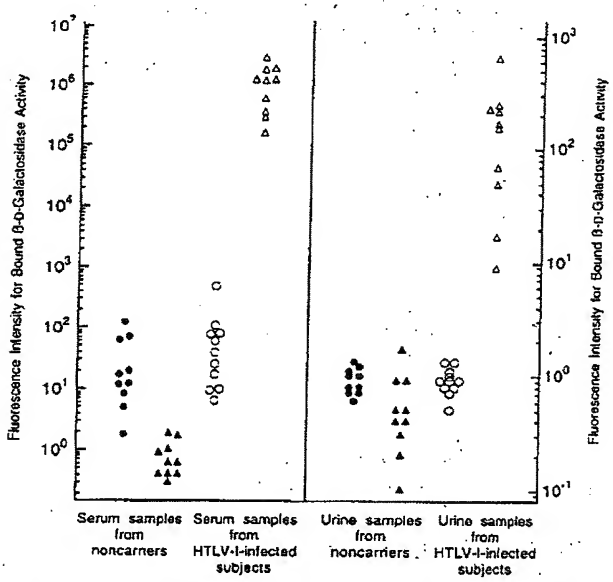

Fig. 7. Cross-reaction test of the immune complex transfer enzyme immunoassay for anti-HIV-1 IgG with serum and urine samples from HTLV-I-infected subjects. Closed and open symbols indicate results with samples from noncarriers and HTLV-I-infected subjects, respectively. Circles and triangles indicate results by the immune complex transfer enzyme immunoassay for anti-HIV-1 IgG and the immune complex transfer enzyme immunoassay for anti-HTLV-I IgG, respectively.

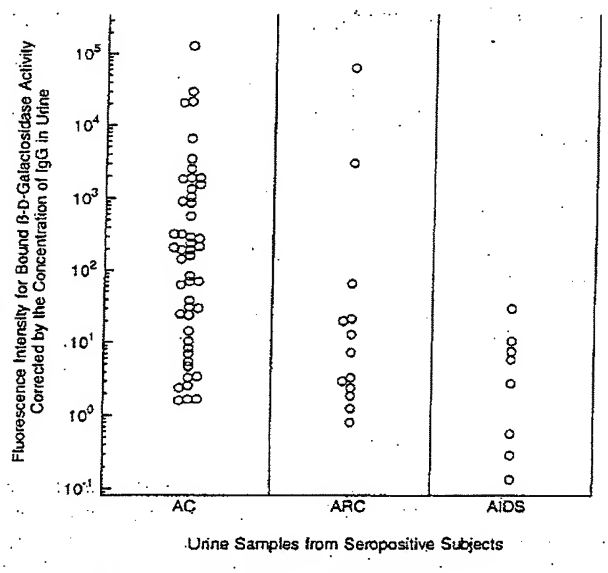

Fig. 8. Level of anti-HIV-1 IgG in urine samples from seropositive subjects at different stages of HIV-1 infection measured by the immune complex transfer enzyme immunoassay. Fluorescence intensities for bound β-D-galactosidase activity were corrected by the concentration of IgG in urine. AC, asymptomatic carriers. ARC, patients with AIDS-related complex. AIDS, patients with AIDS.

ELISA performed using various commercial kits were 93–100% and 92–100%, respectively (20, 21). In only one report, both the sensitivity and specificity were 100%, but the ratio of signal to noise was not improved by concentration of urine samples (22). The sensitivity and specificity of GACELISA were 88.1–99.4% and 97.9–100%, respectively (23–25). Those of GACPAT were 92.5–100% and 97.9–99.6%, respectively (23, 24). In the last two methods, the sensitivity would not be improved by using concentrated urine samples, as long as the level of IgG in urine samples reached the low level needed to saturate the assay anti-IgG binding sites (23).

We also examined the sensitivity and specificity of the conventional ELISA using five recombinant proteins as antigens and gelatin particle agglutination test using a lysate of HIV-1 as antigen (Table 1) (4). Urine samples tested were the same ones used in this study. The sensitivity and specificity of the conventional ELISA were 91% and 99%, respectively, with unconcentrated urine samples and 97% and 57%, respectively, with approximately 10-fold concentrated urine samples. The specificity was considerably lowered by concentration of urine samples, since nonspecific signals for urine samples from the seronegative subjects were markedly enhanced. The sensitivity and specificity of the gelatin particle agglutination test were 83% and 97%, respectively, with unconcentrated urine samples and 97% and 76%, respectively, with approximately 10-fold concentrated urine samples. Even for the 49 asymptomatic seropositive subjects, the sensitivity and specificity of the conventional ELISA with unconcentrated urine samples (90% and 99%, respectively) and those of the gelatin particle agglutination test with unconcentrated urine samples (86% and 97%, respectively) were similar to those for the 70 seropositive subjects including the patients with ARC and AIDS. The lowest signal for the asymptomatic seropositive subjects by the conventional ELISA was only one third of the highest nonspecific signal for the seronegative subjects (Fig. 9).

The previous immune complex transfer enzyme immunoassays using recombinant RT and p17 of HIV-1 as antigens and horseradish peroxidase and *Escherichia coli* β-D-galactosidase, respectively, as labels were superior to the conventional ELISA and gelatin particle agglutination test (4). Urine samples tested were the same ones used in this study. The specificity of test results was confirmed by preincubation with excess of recombinant RT or p17 of HIV-1. The sensitivity and specificity were both 100% with unconcentrated urine samples (Table 1). With recombinant RT as antigen and unconcentrated urine samples, the lowest cut-off indices for the asymptomatic seropositive subjects and the patients with ARC and AIDS were 1.5, 1.4, and 1.1, respectively. With re-

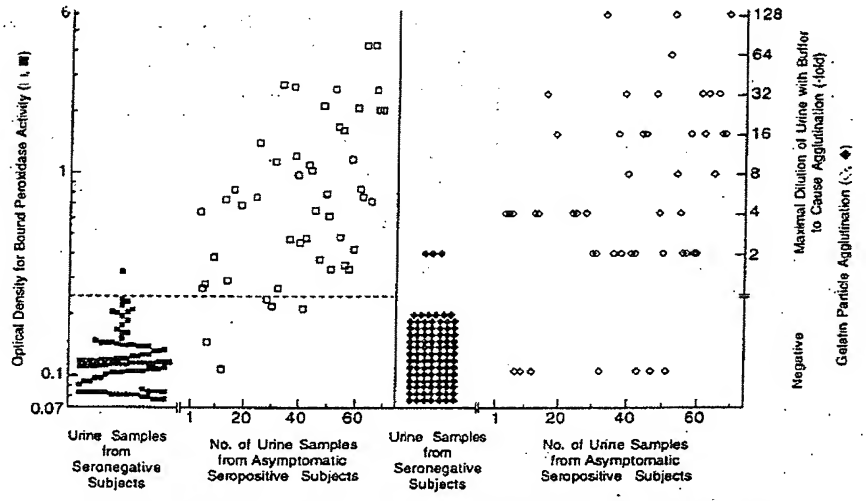

Fig. 9. Detection of anti-HIV-1 IgG or anti-HIV-1 antibodies in urine samples of asymptomatic seropositive subjects by conventional methods. Forty nine urine samples from asymptomatic seropositive subjects and 100 urine samples from seronegative subjects were tested by the conventional ELISA using five recombinant proteins of HIV-1 as antigens and gelatin particle agglutination test using a lysate of the whole virus as antigen. Open and closed symbols indicate results for urine samples from seropositive subjects and seronegative subjects, respectively. Squares and rhombuses indicate results by the conventional ELISA and gelatin particle agglutination test, respectively. The broken line indicates a tentative cut-off value.

combinant p17 as antigen and unconcentrated urine samples, the lowest cut-off indices for the asymptomatic seropositive subjects and the patients with ARC and AIDS were 3.1, 2.6 and 1.9, respectively. In addition, the sensitivity of the immune complex transfer enzyme immunoassay could be improved with concentrated urine samples with no loss of the specificity (100%), since the immune complex consisting of 2,4-dinitrophenyl-antigen, antibody IgG to be detected and antigen-enzyme-conjugate was transferred from one solid phase to another to eliminate interfering substance(s) (Fig. 1) and, as a result, nonspecific signals for seronegative subjects did not significantly change by concentration of urine samples. Therefore, the positivity could be confirmed with concentrated urine samples. Using approximately 10-fold concentrated urine samples, the lowest cut-off indices were significantly improved (3.8, 11, and 2.5 for the asymptomatic seropositive subjects and the patients with ARC and AIDS, respectively, with recombinant RT as antigen and 7.3 and 3.0 for the patients with ARC and AIDS, respectively, with recombinant p17 as antigen).

By the present immune complex transfer enzyme immunoassay using recombinant p24 as antigen with unconcentrated urine samples, the lowest cut-off indices for the asymptomatic seropositive subjects and the patients with ARC and AIDS were 3.1, 2.1, and 2.1, respectively, as describe above. Therefore, test results by the previous immunoassay could be confirmed by the present immunoassay. Namely, the use of p24 as antigen made test results with RT and p17 as antigens more reliable.

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